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EXPERIMENTAL STUDY

Tetrahydroxy stilbene glucoside improved the behavioral disorders of APP695V717I transgenic mice by inhibiting the expression of Beclin-1 and LC3-Ⅱ

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Abstract

OBJECTIVE: To observe the effect of tetrahydroxy stilbene glucoside (TSG) on the behavior of APP695V717I transgenic mouse models and the expression of autophagy-associated proteins Beclin-1 and LC3-Ⅱ.

METHODS: Forty 3-month-old APP695V717I transgenic mice were randomized equally into either a TSG group (n = 20) or a model group (n = 20). A normal control group consisted of C57BL/6J mice of the same age and background (n = 20). The TSG group received TSG intragastric administration for 1 month. Behavior was measured using the Morris water maze and the Y-maze tests. Changes in protein expression and mRNA of autophagy-associated Beclin-1 and LC3-Ⅱ in mice hippocampus were detected by western blot and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analyses.

RESULTS: The number of electric-stimulus escapes significantly increased and the Morris water maze test showed prolonged escape latency, greater swimming distance, less time taken to cross the exact former platform location in the model group, and increased mRNA and protein expressions of Beclin-1 and LC3-Ⅱ compared with the control group ($P < 0.05$). The TSG group showed a decrease in the number of electric-stimulus escapes, shorter escape latency and swimming distance, greater time taken to cross the exact former platform location, and decreased mRNA and protein expressions of Beclin-1 and LC3-Ⅱ compared with the model group ($P < 0.05$).

CONCLUSION: These results indicate that tetrahydroxy stilbene glucoside can decrease expressions of Beclin-1 and LC3-Ⅱ in the autophagy pathway. It can attenuate injury to endoplasmic reticulum functions caused by Ab neurotoxicity, improving learning, memorizing, and spatial orientation behavior in mice.

Key words: Mice, tansgenic; Becn1 protein; LC3 protein; Tetrahydroxy stilbene glucoside; Behavior

INTRODUCTION

As a common degenerative disease of the nervous system, Alzheimer's disease (AD) features a large amount

of senile plaque in brain tissues[1] caused mainly by abnormally-folded b-amyloid protein (Ab). For such abnormally-folded protein in the cell, degradation of proteins and organelles is regulated through the ubiquitin-proteasome pathway and the autophagic lysosome pathway to ensure normal protein and cellular functions. Early research demonstrated the expression of accumulation of many vesicles as a pathological mechanism in AD patients, without a clear description of the expression mechanism and its effects on AD pathogenesis. In 2006, researchers established a mouse model for gene knockout of key proteins Apg5 and Apg7 in the autophagy pathway. This model has enabled the expression of some neurodegenerative disease phenotypes, and drawn attention to the role of the autophagic lysosome pathway in neurodegenerative diseases.[2,3] In this experiment, APP695V717I transgenic AD mouse modelswere used toexamine the expression and significance of autophagy-associated proteins Beclin-1 and LC3-Ⅱ in the hippocampus.

AD involves a gradual loss of learning, memory, and the ability to work, and is an increasingly heavy burden on families and society. However, current medical treatment is not effective. Research shows that 2,3,5,4-tetrahydroxy stilbene-2-2glycoside (tetrahydroxy stilbene glucoside, TSG), the main active ingredient of Heshouwu (*Radix Polygoni Multiflori*), can help to reduce senile plaque deposition induced by b-amyloid. It can improve the learning and memorizing ability of mice,[4] and reduce the expression of amyloid precursor protein (APP).[5] In this paper, we examine the expression of autophagy-associated proteins Beclin-1 and LC3-Ⅱ induced by Ab1-42 and discuss its significance.

MATERIALS AND METHODS

Grouping of experimental mice

Forty 3-month-old APP695V717I transgenic mice comprising20 females and 20 males weighing (30 ± 4) g were selected, together with a normal control group of 20 C57BL/6J mice (10 females and 10 males) of the same age and background. They were all purchased from the Research Center for Laboratory Animal Science, Chinese Academy of Medical Sciences (Certificate No. SCKK 20080013, Beijing, China). The transgenic mice were randomized equally into two groups using a random number table: TSG group (n = 20) and model group (n = 20). The TSG group received 100 mg/kg TSG intragastric administration for 1 month, once per day. The control and model groups received 0.9% physiologicalsaline (Guojing, China) intragastric administration (5 mL/kg per day). All animal-handling and proceduresweremperformed accordingto the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the experimentalanimal ethics committee of Lanzhou General Hospital, Lanzhou Military Area Command (Lanzhou, China).

Reagents and drugs

First antibodies for Beclin-1 and LC3-Ⅱ were purchased fromStressgen Biotechnologies Corporation (Victoria, Canada). The self-designed primer was synthesized by BGI-Shanghai (Shanghai, China) and Trizol, Taq, and RT-PCR were purchased from MBL International Corporation (Woburn, MA, USA). The TSG was a dry powder extracted and separated from Heshouwu (*Radix Polygoni Multiflori*), with a content of 68% (supplied by Hunan Academy of Chinese Medicine, Hunan, China), and it was soluble in water to a concentration of 100 mg/mL.

Behavior detection

(a) The following tests were used. Y-maze electric shock test: the number of electric shock stands (related to learning and memorizing ability), and the number of times each mouse escaped the electric stimulus were recorded, with a maximum frequency of 30. (b) Morris water maze place navigation test (PNT): training wascarried out between 08:00 and 11:00 every day by placing each mouse into the water in a clockwise direction, with the mouse facing the pool wall and without selecting the quadrant of the platform as the place of entry, so that the hidden platform escape latency of the mice was detected. In case of failure to reach the platform within the specified maximum period of 120 s, the operator would guide the mouse to the platform and an escape latency of 120 s was recorded. After a 20 s rest on the platform, the mouse was given the next test. The escape latency, swimming distance, and swimming track for the mouse to find and climb onto the platform were observed and recorded. After completion of training over 3 days, the mouse model was established for the group. PNT was implemented 21 days after establishment of the model. (c) Morris water maze spatial probe test: the platform was removed after the last PNT for each group. The mouse was put into the water at the last place of entry, with the mouse facing the pool tank, and was kept swimming in the water maze. The time taken for the mouse to cross the former platform location within 120 s was recorded.

Ultra-structure detection

Ten mice were selected from each group after the completion of the behavioral test on the 30th day. Intraperitoneal anesthesia was given by 2.5 mL/kg 10% chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA) and decollation was carried out. The brain was taken after quick cardiac perfusion by normal saline and washing, and the hippocampal tissue was stripped on dry ice. After being separated, hippocampal tissues were washed twice with phosphate buffer solution, fixed with 4% glutaraldehyde, and cut into pieces after cooling. Then they were fixed with 2% osmium tetroxide, dehydrated, soaked, and embedded in gradient acetone, and dual stained with uranyl acetate-lead citrate. A transmission electron microscope (TEM) was used for observations.

Measurement of mRNA expressions with RT-PCR

Primers were self-designed on the basis of the gene sequencein GenBank, including upstream primer 5′-CCCTACAGGATG-GATGTGGAGAAAG-3′ and downstream primer 5′-ATTGTGAGGACAC-CCAAGCAAGACC-3′ for the target gene Beclin-1 (162 bp); upstream primer 5′-ACCAAGCCTTC-TTCCTCC-3′ and downstream primer 5′-TGTCC-CGAATGTCTCCTG-3′ for the target gene LC3-II (136 bp); and upstream primer 5′-CGTTGACATCC-GT-AAAGACCTCTA-3′ and downstream primer 5′-TAAAACGCAGCTCAGTAACAGTCCG- 3′ for internal control b-action (100 bp). After agarose gel electrophoresis, the product was observed and photographs were taken under a UV projection lamp.

Measurement of protein expressions with the western blot method

After protein extraction using the density gradient centrifugation method, hippocampal tissue was transferred to the homogenizer after being homogenized by adding lysate, and centrifugation for 10 min (14 000 ×*g*) was performed to take the supernatant. Centrifugation was continued for 15 min (12 000 ×*g*), and the supernatant was taken. Then centrifugation for another 30 min (12 000 ×*g*) was performed. Lysate and protease inhibitor were added, and protein concentration was measured with a bicinchoninic acid protein assay kit. The sample was mixed with buffer solution, boiled for denaturation, and separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) . The proteins were transferred to a nitrocellulose membrane, closed with 5% skim milk, and incubated with first antibody. Horseradish peroxidase was added to mark the incubation with second antibody. An enhanced chemiluminescence kit was used for chemiluminescence detection and images were scanned with a JS-300 gel imager for subsequent analysis and treatment.

Statistical method

The data were processed with SPSS 12.0 statistical software package (SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as the mean ± standard deviation ($\bar{x} \pm s$), *t*-test for comparison between groups, and variance analysis for comparison among multiple groups of independent samples. $P < 0.05$ was considered significant.

RESULTS

Y-maze detection

Compared with the control group, the number of electric-stimulus escapes in the model group significantly increased ($P < 0.01$). After TSG intervention, the number of electric-stimulus escapes in the TSG group significantly decreased compared with the model group ($P < 0.01$, Table 1).

Table 1 Comparison of behavior of mice in Y-maze test ($\bar{x} \pm s$)

Group	*n*	Before modeling	One month later
Control	20	16.9±2.6	17.0±2.2
TSG	20	17.0±2.0[a]	20.1±2.2[ab]
Model	20	16.1±4.4[a]	28.2±3.5[a]

Notes: the control and model groups were treated with 0.9% (physiological) saline (5 mL/kg per day). The TSG group was treated with tetrahydroxy stilbene glucoside (100 mg/kg) for 30 days. TSG: tetrahydroxy stilbene glucoside. Compared with control group, [a]$P < 0.01$; compared with model group, [b]$P < 0.05$.

Morris water maze performance

Compared with the control group, the swimming latency of mice in the model and TSG groups was prolonged ($P < 0.01$), the swimming distance greater ($P < 0.01$), and the time taken to cross the former platform location significantly lower ($P < 0.01$). These differences increased with time. Compared with the model group, latency was shorter ($P < 0.01$), swimming distance was shorter ($P < 0.05$), and the time taken to cross the former platform location greater ($P < 0.01$) for mice in the TSG group (Table 2).

Autophagic lysosome under TEM in CA_1 area of hippocampus of mice

Control group:complete nerve cell membrane, uniform electron density of cytoplasmic matrix, visible intracytoplasmic mitochondria, abundant intracytoplasmic mitochondria and rough-surfaced endoplasmic reticulum with roughly normal structure and clear and complete mitochondrial cristae, round nucleus and complete and clear nuclear membrane, unchanged Golgi apparatus (Figure 1A). TSG group: clear intracytoplasmic mitochondria, with a small number of visible mitochondrial cristae disappearing and few vacuolar degenerations, clear rough-surfaced endoplasmic reticulum with a few showing slight expansion, and occasional cy-

Table 2 Comparison of behavior of mice in Morris water maze test ($\bar{x} \pm s$)

Group	*n*	Latency (s)	Distance (m)	Platform crossing (times)
Control	20	17.82±2.13	3.87±1.12	18.52±3.04
TSG	20	25.18±2.76[ab]	6.74±2.05[ab]	3.92±1.17[ab]
Model	20	31.65±3.11[a]	8.46±2.13[a]	3.18±1.09[a]

Notes: the control and model groups were treated with 0.9% (physiological) saline (5 mL/kg per day). The TSG group was treated with tetrahydroxy stilbene glucoside (100 mg/kg) for 30 days. TSG: tetrahydroxy stilbene glucoside. Compared with control group, [a]$P < 0.01$; compared with model group, [b]$P < 0.05$.

toplasmic edema, complete nuclear membrane, normal Golgi apparatus (Figure 1B). Model group:unclear intracytoplasmic mitochondria, without cristae, a small number of them in vacuolar degeneration, unclear rough-surfaced endoplasmic reticulum, with some expanded and with unclear nuclear membrane, roughly normal Golgi apparatus, formation of a large amount of visible autophagic lysosome (Figure 1C).

mRNA and protein expressions of Beclin-1 and LC3-II in hippocampus

As shown in Figures 2 and 3, for the RT-PCR product with agarose gel electrophoresis and western blot analysis, mRNA and protein expressions of Beclin-1 and LC3-Ⅱ in the model group showed a consistent tendency, which was significantly different from that of the control group ($P < 0.05$). The mRNA and protein expressions of Beclin-1 and LC3-Ⅱ in the TSG group were significantly lower than in the model group ($P < 0.05$, Table 3).

DISCUSSION

The results of western blot and RT-PCR analyses showed a significant increase in both LC3-Ⅱ and Beclin-1 expression in the hippocampus of transgenic mice, indicating that Ab produced by the mice activated the autophagic lysosome pathway. It is presumed that during fibrosis formation, neurotoxicity produced by Ab can result in functional disorder of the endoplasmic reticulum; however, the endoplasmic reticulum self-regulation system can balance cellular stress with

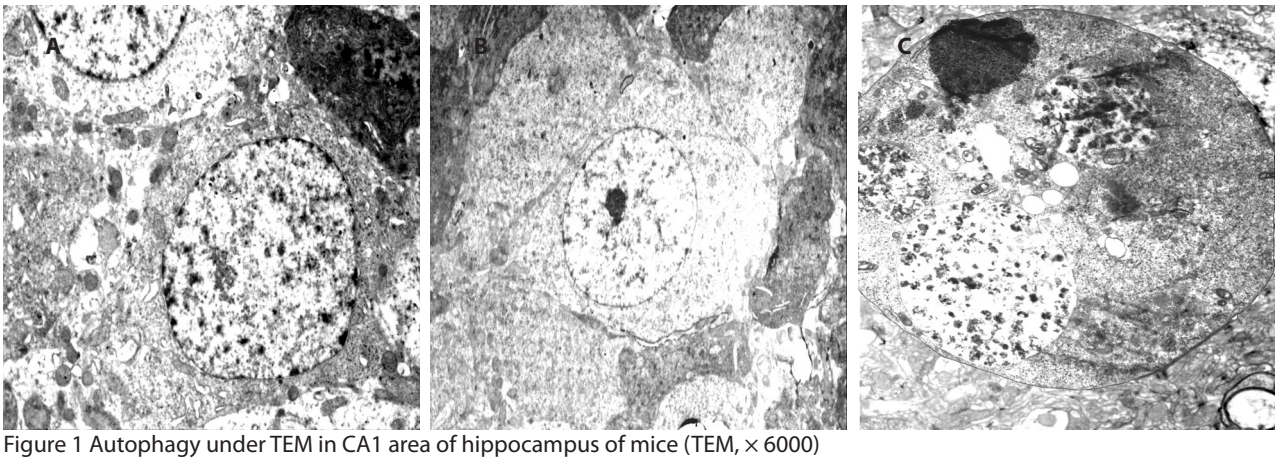

Figure 1 Autophagy under TEM in CA1 area of hippocampus of mice (TEM, × 6000)
A: control group; cell structure is normal and uranyl acetate-lead citrate is dual stained. B: TSG group; cell structure is mildly abnormal and uranyl acetate-citrate lead is dual stained. C: model group; cell structure has some changes, a large number of autophagic vacuoles are formed, and uranyl acetate- lead citrate is dual stained. The control and model groups were treated with 0.9% (physiological) saline (5 mL/kg per day). The TSG group was treated with tetrahydroxy stilbene glucoside (100 mg/kg) for 30 days. TSG: tetrahydroxy stilbene glucoside; TEM: transmission electron microscope.

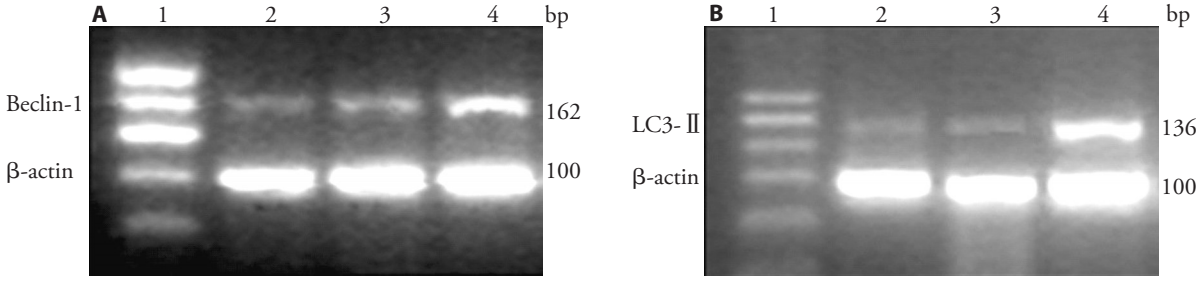

Figure 2 mRNA levels of Beclin-1 and LC3-Ⅱ in hippocampus
A: changes of Beclin-1 mRNA levels in the hippocampus of mice; B: changes of LC3-IImRNA levels in the hippocampus of mice. 1: maker; 2: control group; 3: TSG group; 4: model group. The controland model groups were treated with 0.9% (physiological) saline (5 mL/kg per day). The TSG group was treated with tetrahydroxy stilbene glucoside (100 mg/kg) for 30 days. TSG: tetrahydroxy stilbene glucoside.

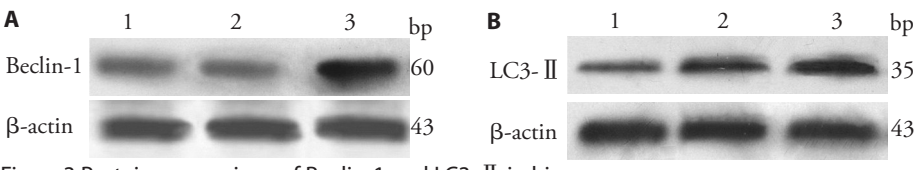

Figure3 Protein expressions of Beclin-1 and LC3-Ⅱ in hippocampus
A: western blot analyses of expression levels for Beclin-1 in the hippocampus of mice; B: western blot analyses of expression levels for LC3-Ⅱ in the hippocampus of mice. 1: control group; 2: TSG group; 3: model group. The controland model groups were treated with 0.9% (physiological) saline (5 mL/kg per day). The TSG group was treated with tetrahydroxy stilbene glucoside (100 mg/kg) for 30 days. TSG: tetrahydroxy stilbene glucoside.

Table 3 mRNA and relative protein expressions of Beclin-1 and LC3-II for mice (%, $\bar{x} \pm s$)

Group	*n*	Relative expressions of mRNA		Relative expressions of protein	
		Beclin-1	LC3-Ⅱ	Beclin-1	LC3-Ⅱ
Control	20	0.247±0.013	0.230±0.027	0.200±0.012	0.258±0.018
TSG	20	0.408±0.019[ab]	0.335±0.008[ab]	0.487±0.017[ab]	0.361±0.021[ab]
Model	20	0.576±0.011[a]	0.417±0.013[a]	0.632±0.008[a]	0.516±0.009[a]

Notes: the control and model groups were treated with 0.9% (physiological) saline (5 mL/kg per day). The TSG group was treated with tetrahydroxy stilbene glucoside (100 mg/kg) for 30 days. TSG: tetrahydroxy stilbene glucoside. Compared with control group, [a]$P < 0.05$; compared with model group, [b]$P < 0.05$.

intracellular autophagic lysosome.

Some experts have suggested that autophagic lysosome is an important process for production of toxic Ab40 or Ab42. During *in vivo* and *in vitro* tests, Yu *et al* [6] and LeBlanc *et al* [7] discovered that Ab existed in autophagosomes, as well as in prosomes of bCTF, APP and g-secretes; after improvement of autophagic lysosome function with rapamycin or serum deprivation, g-secretes can be transferred from the endoplasmic reticulum to the autophagosome, with greatly increased production of Ab. Haass *et al* [8]discovered that intracellular Ab damaged cells significantly more than did extracellular Ab. Additionally, the Ab produced through the autophagic lysosome pathway can be degraded with lysosomes, and can be secreted outside the cell to form insoluble abnormal aggregates. All these results indicate that failure of the autophagic lysosome pathway may cause increased toxic Ab that will in turn damage neurons and lead to the formation of extracellular senile plaque.

Earlier experiments revealed that Ab-induced endoplasmic reticulum stress and related apoptosis pathways are associated with injury to brain neurons through Ab neurotoxicity.[9] Current research indicates that during endoplasmic reticulum stress, the protein kinase R-like endoplasmic reticulum kinase (PERK) signal pathway causes accumulation of a large number of proteins, including polyglutamine poly Q72, in the endoplasmic reticulum, which inhibits related endoplasmic reticulum degradation systems. Moreover, phosphorylated eIF2a and increased autophagic lysosome protein l2 (Atgl2) form compound Atg5-Atgl2-Atgl6, and cause LC3-Ⅱ membrane translocation, inducing autophagic lysosome.[10] Both previous research and the present findings suggest that when Ab is secreted outside the cell, the neurotoxicity shown during formation of fibrosis begins to take effect; Ab can destroy cell membrane-induced oxidative stress, which in turn can destroy endoplasmic reticulum functions. Within a short period of time, the induced endoplasmic reticulum stress can provide protection against damage to the brain by activating unfolded protein protective pathways, such as PERK, and can increase the probability of misfolding of intracellular proteins in the endoplasmic reticulum, causing abnormal shear and accumulation of proteins. Proteins with abnormal shear can activate the LC3-Ⅱ and Beclin-1 autophagic lysosome pathway of the cell, but when autophagic lysosome fails to degrade excess proteins, cell death may be induced by activation of the endoplasmic reticulum-specific caspase-12 apoptotic pathway by excessive stress. Therefore, extracellular Ab can induce more intracellular abnormal shear and protein deposition, and such misfolded proteins can not only induce programmed cell death, but also induce cellularautophagic lysosome, leading to progressive worsening of behavioral disorders.

Research into Heshouwu (*Radix Polygoni Multiflori*) shows that it improves the learning and memorizing ability of mice in the AD model, and protects cerebral cholinergic neurofibers, improving the activity of acetylcholine esterase and inhibiting apoptosis through regulation of the apoptosis-related Bax and Bcl-2 pathway.[11-14] Future TSG research may reveal the main mechanism of action and drug targets for AD treatment.

With TSG intervention, the escape latency and swimming distance for mice were shortened, but the time taken to cross the former platform location was greater, indicating that TSG can improve learning, memory, and spatial orientation in mice, and has protective effects on the brain. Western blot and RT-PCR analyses showed that after intervention with TSG, mRNA and protein expressions of LC3-Ⅱ and Beclin-1 in the hippocampus decreased.

We conclude that the extract TSG from Heshouwu (*Radix Polygoni Multiflori*) can significantly improve learning, memorizing, spatial orientation, and other behavioral functions in transgenic mice. Its main mechanism may be to inhibit the expression of Beclin-1 and LC3-Ⅱ in autophagic lysosome pathways. Thisprotects the endoplasmic reticulumfunctions from Aβneurotoxicity and improves behavioral disorders intransgenic mice. It is likely that TSG will become an effective treatment to improve cognitive function of patients with AD.

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